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Novel, Nonconsensus Cellular Splicing Regulates Expression of a Gene Encoding a Chemokine-like Protein That Shows High Variation and Is Specific for Human Herpesvirus 6

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There are few genes that are specific and diagnostic for human herpesvirus-6. U83 and U22 are two of them. U22 is unique, whereas U83 encodes distant similarity with some cellular chemokines. Reverse transcription-polymerase chain reaction, cDNA cloning, and sequence analyses show polyadenylated RNA transcripts corresponding to minor full-length and abundant spliced forms of U83 in human herpesvirus 6-infected cells. The splice donor and acceptor sites do not fit consensus sequences for either major GT-AG or minor AT-AC introns. However, the spliced form can also be detected in a U83 transfected cell line; thus the novel sites are used by cellular mechanisms. This intron may represent a new minor CT-AC splicing class. The novel splicing regulates gene expression by introducing a central stop codon that abrogates production of the chemokine-like molecule, resulting in an encoded truncated peptide. The use of metabolic inhibitors and an infection time course showed expression of the two RNA transcripts with immediate early kinetics. However, the full-length product accumulated later, dependent on virus DNA replication, similar to U22. Sequence analyses of 16 strains showed high variation (13%) in U83 , with conservation of the novel splice sites. Representative strain variants had similar kinetics of expression and spliced products.

INTRODUCTION

The prevalence of human herpesvirus 6 (HHV-6) is widespread, and it is one of the more recently identified human herpesviruses. Herpesviruses cause persistent/latent infections for the lifetime of the host and can reactivate during immunosuppression to cause disease. HHV-6 is a large double-stranded (ds)DNA virus (159 kb) encoding more than 100 genes; it is closely related to human herpesvirus 7 (HHV-7) and shares genomic similarity with other betaherpesviruses, like human cytomegalovirus (HCMV) (Gompels et al., 1995; Nicholas, 1996). HHV-6 and HHV-7 cause pediatric febrile illness with occasional severe or fatal complications (Asano *et al.,* 1990, 1992; Hall *et al.,* 1994; Portolani *et al.,* 1997). In immunocompromised patients such as transplant or AIDS patients, the viruses can reactivate; here the better-defined HHV-6 is associated with more severe disease, including bone marrow suppression and encephalitis (Asano *et al.,* 1990, 1992; Drobyski *et al.,* 1993, 1994; Hall *et al.,* 1994; Knox *et al.,* 1995; Wang *et al.,* 1996; Portolani *et al.,* 1997). A link with multiple sclerosis has been suggested (Challoner *et al.,* 1995; Soldan *et al.,* 1997). Together with human immunodeficiency virus (HIV), HHV-6 and HHV-7 have a cellular tropism for $CD4^+$ T lymphocytes, which they infect and kill via cell fusion and lysis (Takahashi *et al.,* 1989; Lusso *et al.,* 1994). There is some evidence for interaction between these viruses in lytic or reactivating latent infections *in vitro*. *In vivo*, most evidence indicates HHV-6, including disseminated and fatal infections in HIV/AIDS patients, as well as a possible factor in their progression (Emery *et al.,* 1999; Knox and Carrigan, 1994, 1996).

Herpesvirus gene expression is tightly regulated during lytic and latent infections. Limited data suggest this also applies to HHV-6. In latent infections, there is little virus gene expression, whereas in lytic infections, there is a cascade of expression from immediate-early (IE) to early to late times postinfection (p.i.). IE genes require no prior virus gene expression, whereas late genes require both virus gene expression and DNA replication (Mirandola *et al.,* 1998). At least one IE gene, Rep or U94, appears to also be expressed during *in vitro* or *in vivo* latent infections and shows suppression of virus DNA replication (Rotola *et al.,* 1998; S. Turner, D. DiLuca, and U. A. Gompels, unpublished results). In general, herpesviruses have relatively few spliced genes compared with the host cell, and spliced genes usually are either rare latent genes or of the IE kinetic class with genes expressed later under virus gene regulation usually lacking splicing. However, much of these data relate to herpes simplex virus type 1 (HSV1) or HCMV (Mocarski *et al.,* 1990; Wagner *et al.,* 1995; Meier and Stinski, 1996). Little

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is known about regulation in HHV-6, and preliminary data show up-regulation of the cell cycle rather than the host cell shutoff as observed in HSV1 (Black *et al.,* 1991). Recently, complex patterns of splicing have been documented in HHV-6-infected cells in both IE and late transcripts, which suggests there may be control of splicing or splicing efficiency that may be different from that of the other herpesviruses (Mirandola *et al.,* 1998). Comparisons of complete genomic sequences of different HHV-7 strains with HHV-6 and HCMV suggest there may be more splicing in these betaherpesviruses than currently recognised (Gompels *et al.,* 1995; Megaw *et al.,* 1998). To date, splicing observed in herpesviruses, including those reported for HHV-6, conform to the major splice class as represented in spliced gene catalogues (Mount, 1982; Stephens and Schneider, 1992). Here we investigate expression and aberrant splicing of genes specific to HHV-6.

Most HHV-6 genes have homologues in the closely related HHV-7, but a few are specific and diagnostic for HHV-6 infections. One gene is U22, which encodes a specific glycoprotein unique to HHV-6. Another specific gene is U83, which was identified in genomic sequencing studies as the first viral gene to encode distant sequence similarity to chemokines (Gompels *et al.,* 1995). These proteins are proinflammatory cytokines that can attract specific leukocyte subsets to a site of infection (Luster, 1998). Some chemokines can also inhibit certain strains of HIV via interactions with the chemokine receptors that act as HIV coreceptors with CD4 (Cairns and D'Souza, 1998). Thus expression of U83 may be important to the specific biology of HHV-6. Other herpesviruses encode chemokine-like proteins. Human herpesvirus 8 (HHV-8), the Kaposi's sarcoma-associated herpesvirus, encodes two chemokine homologues that can inhibit HIV infection; at least one can bind chemokine receptors (Boshoff *et al.,* 1997). Murine cytomegalovirus encodes a protein with sequence similarity to chemokines and is expressed as a late gene (MacDonald *et al.,* 1997). The temporal regulation of these chemokine-like genes has important implications for the replicative cycle of the virus in possible contributions to immune evasion or participation in virus spread.

In this report, we investigate the expression of U83 and show that it is regulated by novel splicing. The results show U83 is produced as two mRNA transcripts. One transcript corresponds to the full-length U83 gene, whereas the other, more abundant message is interrupted by splicing, with an intron of 77 nucleotides excised and a stop codon introduced. We show that the U83 splice acceptor and donor sites do not conform to currently defined consensus sites. We demonstrate that the novel splicing is not cell specific or virus dependent. Production and accumulation of the full-length transcript do, however, appear to require a virus or virus-induced component. Kinetics data are described illustrating the spliced transcript with characteristics of the IE class, produced in the absence of virus protein production, whereas the unspliced U83 product is maximally produced late after virus DNA replication, similar to the kinetics shown for U22. The effects of strain variation are assessed in relation to the novel splicing. The results show different levels of gene regulation for the production of the viral chemokine-like protein and suggest the existence of a novel cellular mechanism for control of gene expression.

RESULTS

The U83 gene is expressed as full-length and spliced forms in HHV-6-infected cells

Comparisons between HHV-6 and the closely related HHV-7 show that there are homologues for most of the genes. There are a few genes that are specific for HHV-6 that encode properties distinct for this virus. U83 encodes a small chemokine-like protein (Gompels *et al.,* 1995), one of HHV-6-specific genes that is diagnostic for this virus. To examine the expression of this potentially important gene in HHV-6, a series of reverse transcription-polymerase chain reaction (RT-PCR) analyses were undertaken. Initially the prototype strain U1102 (variant A strain group) was used; then all results were repeated using strain CV as a B variant representative. The mRNA transcript was characterised by RT-PCR of poly(dT)-generated cDNA from U1102-infected JJhan (Jurkat clone, T-leukemic) cells 5 days p.i. The production of all cDNA was monitored by amplification of the β-actin gene (data not shown). U83-specific primers, P7 and U83C, were designed from the HHV-6 genomic sequence (strain U1102) to amplify the entire coding sequence together with 5′ and 3′ untranslated regions, resulting in a 402-bp product. However, the RT-PCR results showed only very weak amplification of the 402-bp product (optimised in later kinetic studies described below) but strong amplification of a smaller product of ~300 bp (Fig. 1A). To show this was not an artifact of a single primer set, amplification of the U83 transcript was repeated using further U83 gene-specific primer pairs shown in Fig. 1 (P6 and U83C, U83F2 and U83C, U83P1 and U83P2, and U83F and U83B). In all cases, a product, ~100 bp smaller than expected from the DNA sequence, was preferentially amplified (data not shown). To investigate whether these U83 mRNA transcripts were an aberration due to the expression in a leukemic T cell line, identical experiments were performed in primary cells [peripheral blood mononuclear cells (PBMCs)]. Again, the smaller 300-bp product was amplified in greater abundance compared with the expected 402-bp product (Fig. 1B). Thus the amplification of the smaller U83 mRNA transcript is not dependent on the cell type. Sequence analyses of the smaller RT-PCR products from the JJhan cells and the

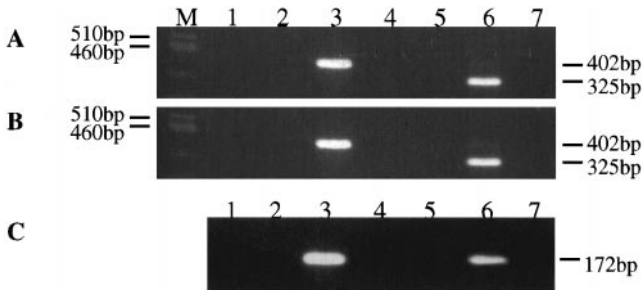

FIG. 1. RT-PCR analysis of HHV-6 U83 from uninfected and U1102-infected cells. RT-PCR products generated using primers U83P7 and U83C, which amplify the whole U83 gene region from (A) infected JJhan cells or (B) infected PBMCs. (C) RT-PCR products generated from infected JJhan cells, using primers U83P7 and U83INB, specific for the full-length transcript by using one intron primer. Lane 1, H_2O negative control; lane 2, uninfected DNA; lane 3, U1102-infected DNA; lane 4, H_2O negative control; lane 5, uninfected cDNA; lane 6, U1102-infected cDNA; and lane 7, H_2O negative control. Sizes of products are in base pairs.

PBMCs showed them to be spliced transcripts of U83, from which an intron of 77 bp had been excised (Fig. 2).

To detect the presence of full-length U83 only and not the spliced U83 transcript, a 3′ intron-specific primer (83INB) was designed. This allowed us to investigate the presence and abundance of any full-length U83 mRNA transcript. The previous RT-PCR results (Fig. 1) show that the full-length transcript, with an expected size of 402 bp, is only just visible in the infected JJhan and PBMC systems. Amplification with the 5′ primer, P7, and the 3′ intron-specific primer, U83INB, on identical cDNA resulted in the full-length U83 transcript being amplified to a detectable level. An identical result was obtained using the PBMC-generated cDNA (data not shown). Thus both full-length and spliced transcripts are expressed from the U83 gene. Identical results were shown for strain CV (variant B strain group) giving a similar spliced product and are described further in the sequencing and kinetic experiments.

Sequence analyses of the U83 transcripts show novel splice sites

The spliced and unspliced products were characterised further by sequence analyses. The RT-PCR products from all primer sets, external primers and intron primers, were gel-purified and cloned into pGEM^{R}T, using TA ligation, followed by nucleotide sequencing. This showed that both a full-length and a spliced polyadenylated transcript of U83 are present in HHV-6-infected JJhan cells and PBMCs. The cDNA encoding the spliced transcript lacks an intron of 77 bp that occurs 121 bp from the U83 ATG start codon, giving a 325-bp product (Fig. 2). The sequence of the unspliced transcript is identical to the published U83 DNA sequence (Gompels *et al.,* 1995), except for a CG inversion that resulted in a coding change (nucleotide 213, amino acids 71/72 DE to EQ); this was confirmed by direct sequencing of PCR products from multiple stocks of U1102, including the earliest passaged isolate. Similar splicing results were shown for an HHV-6 variant B strain CV by nucleotide sequencing of the spliced and full-length products (shown below).

The acceptor and donor splice sites utilised by the U83 intron do not conform to the consensus for the major class of introns, GT-AG (Fig. 3). The sites varied from these major class, U2-type, spliceosomal introns, which have the consensus of (with underlined invariant residues) AG^GURAGU for the donor and YAG^ for the acceptor together with a preceding polypyrimidine tract (where Y is pyrimidine, R is purine, and ^ indicates the splice site) (Mount, 1982; Stephens and Schneider, 1992; Kramer, 1996). Furthermore, the U83 splice sites also do not conform to the minor class, U12-type, of introns, AT-AC, which have recognition donor and acceptor consensus of ^AUAUCUU and YCAC^ of U12-type introns (Hall and Padgett, 1994). Moreover, in U83 there is no recognisable branch site consensus for the minor splicing class, UCCUUAAC, and only a weak match (TCATGAT) for the major class branch consensus of YNYURAY (N indicates any nucleotide) (Kramer, 1996) at 32 bp upstream from the acceptor splice site (Fig. 2B). Given that this may be a novel type of intron, it is not possible to predict the branch site. Even if the intron splicing was mediated by U2 snRNA, it would be difficult to identify the conserved adenine branchpoint for the major splice class given the overall adenine-rich nature of this area.

The exact location of the novel splice site in U83 is not clear because both the donor and acceptor have a direct repeat of TACC. This is similar to the repeated AGG bounding some of the major class splice sites. Although there is some similarity to features of both the major and minor splicing classes, the splice sites in U83 are distinct and do not conform easily to either grouping. Taking the U83 splice preceding CT as the donor and AC in the acceptor, these sites may represent a new minor class of intron, CT-AC.

Rapid amplification of cDNA ends analyses of the 3′ end of the U83 transcripts

The poly(A)$^+$ site available downstream from the U83 ORF overlapped the next ORF, U84, a homolog of a spliced message in HCMV (Gompels *et al.,* 1995). Therefore, rapid amplification of cDNA ends (RACE) was used to examine whether this poly(A)$^+$ site was utilised or whether there was any further splicing at the 3′ end of the cDNA. A poly(dT) 3′ primer was used together with a 5′ primer U83F2 to amplify a RACE product from U1102-infected PBMCs. Sequence analyses showed this product to be the unspliced transcript of U83, again demonstrating its presence in a polyadenylated form (Fig. 2). The 3′ noncoding region of the unspliced U83 cDNA was shown to end 12

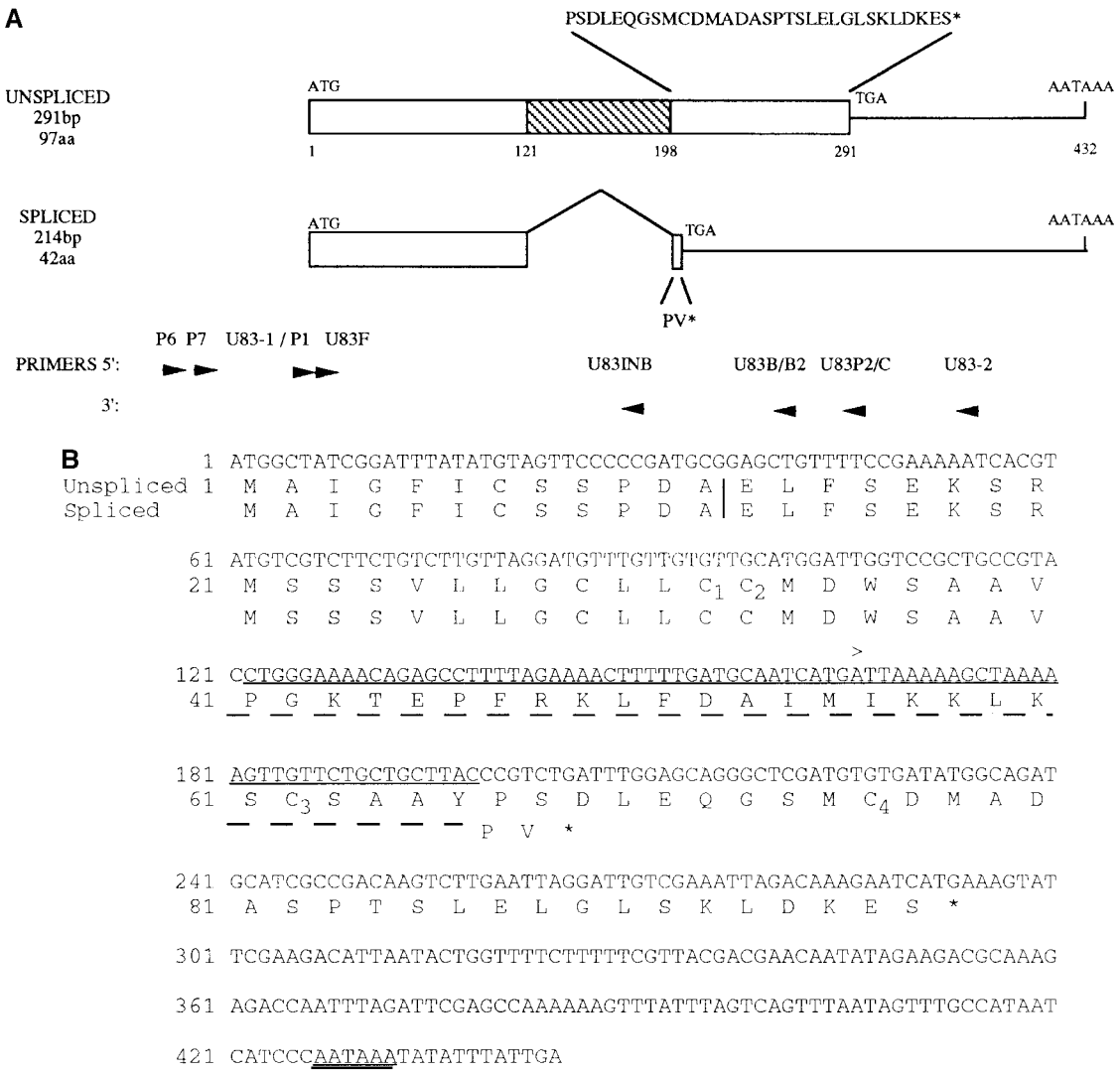

FIG. 2. (A) Summary of the structure of the U83 spliced and unspliced transcripts. Open and shaded areas represent the exons and intron, respectively. AATAAA indicates the location of the polyadenylation signal. The locations of the ATG start codons and TGA termination codons are indicated. The sizes of the transcripts from the start codons are indicated (bp); sizes of the encoded proteins (as determined by the sequence) are also shown (kDa). The determined protein sequence after the intron region is indicated in single-letter amino acid codes for both the unspliced and spliced forms of transcript. Primers used in the analyses are indicated by arrows. (B) DNA and deduced amino acid sequence of the unspliced and spliced U83 transcripts. The predicted signal peptide cleavage point is indicated by a vertical line. The 77-bp intron sequence is underlined. Termination codons are indicated by asterisks. The polyadenylation signal is double-underlined. The chemokine-like signature cysteine residues are numbered 1–4. The dashed line in the spliced protein sequence indicates the sequence has no interruptions.

bases after a typical AATAAA polyadenylation signal, 149 nucleotides after the stop codon (Fig. 2). This indicates that the full-length U83 transcript uses the first polyadenylation site after the stop codon. Using the poly(dT) primer, a smaller product was generated that corresponded to the size of the intron removal with no further differences (not shown). An element for mRNA degradation, TTATTTA, frequently found in the 3′ noncoding regions of cytokines and chemokines (Lagnado *et al.,* 1994) was found to precede the polyadenylation signal, although it is unknown whether this functions in the U83 transcript. Based on the RACE analyses, there was no evidence of further splicing for the U83 ORF in addition to the novel splicing defined above.

The novel U83 splicing stops expression of an encoded chemokine-like protein

The full-length U83 mRNA transcript encodes a putative protein of 97 amino acids, 10.4 kDa in size, with a signal peptide sequence at the N-terminal end. The putative protein contains the characteristic four cysteine residues of β-chemokines (Fig. 2B) as described previously (Gompels *et al.,* 1995). The two putative U83 proteins are shown in Fig. 2B. In the spliced transcript, the

Donors

	P	-4	-3	-2	-1	0	1	2	3	4	5	6	7
	N	136	136	136	136	136	136	136	136	136	136	136	136
	T	28	8	15	17	0	136	9	16	7	84	30	36
	C	41	60	16	7	0	0	3	13	3	17	28	39
	A	40	56	89	12	0	0	83	91	12	23	53	33
	G	27	12	16	100	136	0	41	16	114	12	25	28
CON		N	C	A	G	^ G	T	A	A	G	T	A	N
U83A		G	T	A	C	^ C	T	G	G	G	A	A	A
U83B		G	T	A	C	^ C	T	G	G	G	A	A	G
			@	*	@	@	*		@	*		*	

Acceptors

	P	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3
	N	113	113	113	113	113	113	113	113	113	113	113	113	113	113	113	113	113	113
	T	58	50	57	59	67	56	58	49	47	66	64	31	34	0	0	11	41	31
	C	21	28	34	25	29	33	35	32	42	40	33	25	74	0	0	23	28	41
	A	17	11	11	18	7	17	12	23	15	3	10	29	5	113	0	24	21	21
	G	17	24	11	11	10	7	8	9	9	4	6	28	0	0	113	55	23	20
CON		T	T	T	T	T	T	T	T	T	T	T	N	C	A	G	^ G	N	N
U83A		T	G	T	T	C	T	G	C	T	G	C	T	T	A	C	^ C	C	G
U83B		T	G	T	T	T	T	G	C	T	G	C	T	T	A	C	^ C	T	G
		*		*	*	*	*	@		*	@				*	@	@		

* identity with consensus
@ different, not seen at this position or infrequent.

FIG. 3. Splicing sites do not conform to consensus. (A) Comparisons with a matrix based on Mount's (1982) catalog of major class sites as adapted by Staden (1991) for use in the Nip program for the major class of splice consensus. Sites are shown for U1102 (variant A) and CV (variant B).

intron of 77 bp introduces a single-base frameshift, causing a coding change from serine to a valine, and introducing a TGA stop codon after two codons following the splice acceptor site. This now encodes a shorter peptide of only 42 amino acids, predicted to be 5 kDa in size but identical to the full-length U83 protein at its N-terminal region. The encoded spliced amino acid sequence now contains only two of the four conserved cysteines of the β-chemokine-like motif. To check whether this was an aberration of the prototype strains analysed, the coding sequences of 16 strains were examined.

Sequence analyses of strains show high variation but conserved novel splice sites

Comparisons between amino acid sequences published for HHV-6 prototype variant A strain (U1102) and variant B strain (Z29) show relatively high variation (13%), mostly in the first three fourths of the sequence. To check whether the novel splicing identified in U83 was not just an aberration of the strains analysed, we examined this region in 16 strains by PCR followed by nucleotide sequencing and multiple alignments (Fig. 4). Included were general laboratory reference strains, U1102, Z29, and CV, compared with strains identified in clinical isolates and biopsies from pediatric patients. These strains were from both variant A and B classes of HHV-6 (Gompels *et al.,* 1993; Kasolo *et al.,* 1997). The results confirm up to 10% and 13% nucleotide and amino acid sequence variation, respectively, between all the strains in pairwise comparisons, with up to 3% variation within the variant A or B strain groupings (Figs. 4 and 5). In contrast to U83, similar analyses of the other HHV-6-specific gene, U22, showed conservation between strains from both variant groups with $<$5% variation, a level previously shown for genes such as glycoprotein H that are conserved in herpesviruses rather than specific for an individual virus (not shown) (Gompels *et al.,* 1993).

Despite the relatively high level of variation in U83 for this herpesvirus, the novel splice sites are conserved. There is only one base change at position 200 (C to T, groups A and B, respectively) in the splice acceptor, although this does not appear to affect the splicing as shown by the sequence of the spliced product of the variant B CV strain. Interestingly, there are upstream sites that show some matches to the major class donor consensus and only weak acceptor consensus downstream. The major class donor sites are weakened in the strain variants (Fig. 4). In contrast, in the strains from B variants, the novel splice duplication TACC is extended to TACCTG (Fig. 6). Between the strains, there was additional variation in sequence and length in the 5′ upstream region; however, this was strain specific and did not correlate with the variant A or B grouping (data not shown). Analyses of the encoded amino acid sequence from the strain variants showed high amino acid variation but conservation of the β-chemokine-like motifs and adjoining sequences (Fig. 5). Moreover, in all strains, the conserved splice introduces a stop codon that interrupts the encoded chemokine-like protein between the crucial conserved cysteines and divides it into a truncated, possibly nonfunctional peptide (Fig. 5).

Virus regulation of U83 spliced as an IE gene and full length as a late gene

Having demonstrated expression of both full-length and novel spliced versions of U83 and conservation of the splice sites in the strain groups, we next examined the kinetics of their expression. Herpesvirus genes

```
                    10          20          30          40          50
A1-4      1 ATGGCTATCG GATTTATATG TAGTTCCCCC GATGCGGAGC TGTTTTCCGA
B1        1 ---------- -------CG- ---------- ---------- ---C---A--
B2-3      1 ---------- ----C--TG- ---------- ---------- ---C------

                    60          70          80          90          100
A1-2     51 AAAATCACGT ATGTCGTCTT CTGTCTTGTT AGGATGTTTG TTGTGTTGCA
A3-4     51 ---------- --T------- ---------- ---------- ----------
B1       51 ---T------ --T------- -------A-- ---------- ----------
B2       51 ---T------ --T----T-- -------A-C ---------- ----------
B3       51 ---T------ --T------- -------A-C ---------- ----------

                   110         120         130         140         150
A1-2    101 TGGATTGGTC CGCTGCCGTA CCTGGGAAAA CAGAGCCTTT TAGAAAACTT
A3      101 ---------- ---------- ---------- -----T---- ----------
A4      101 ---------- ---------- ---------- ----AT---- ----------
B1-3    101 CA-------- -----T---- --------G- -----A---- C-------C-

                   160         170         180         190         200
A1      151 TTTGATGCAA TCATGATTAA AAAGCTAAAA AGTTGTTCTG CTGCTTACCC
A2      151 -------A-- ---------- ---------- ---------- ----------
A3      151 ---------G ---------- ---------- ---------- ----------
A4      151 ---------- --C------- ---------- ---------- ----------
B1-3    151 ----T----- ---------- ---------- -------T-- ---------T

                  210    216
A1-4    201 GTCTGATTTG GAGCAG
B1-3    201 ---------A ------


A1=U1102, Z7; A2=Z5; A3=Z23; A4=Z59; B1=Z29, MBE, HT; B2= CV; B3=BOU,
Z3, Z12, Z13, Z14, Z25, Z317
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FIG. 4. Multiple alignments of 14 HHV-6 strains show conservation of novel splice sites. Alignment of the first 210 bases of the U83 gene using the CLUSTALV program (Higgens algorithm) as implemented on a Macintosh computer. Major site donor sites predicted using the Staden program are indicated by underlining the conserved GT, and the TACC repeats of the novel splice donor and acceptor in actual use are also underlined.

can be classified into three kinetic groups of expression: IE, which are produced without *de novo* protein synthesis [resistant to cycloheximide (CEX)]; early genes, which are regulated by the expression of IE genes; and late genes, which are those expressed after DNA replication [sensitive to phosphonoacetic acid (PAA)]. Northern blot analyses of poly(A)$^+$-selected RNA showed IE expression of the U83 transcript. There was insufficient resolution at this size to differentiate expression of the spliced and unspliced products. To examine this in further detail, the kinetics of the full-length and spliced transcripts of U83 were

```
                1                                                   46
HHV-6 U83A  ....MAIGFI CSSPDAELFS EKSRMSSSVL LGCLLCCMDW SAAVPGKTEP
HHV-6 U83B  ....MAIGFI GSSPDAELSS ENSRISSSVL LGCLLCCTDW SAVVPGKTET
HHV-8 Mip1    MDTKGILL VAVLTALLCL QSGDTLGASW HRPDKCCLGY ....QKRPLP
RANTES       MKVSAARLA VILIATALCA .PASASPYSS DTTP.CCFAY IA....RPLP
IL8        MTSKLAVALLA AFLISAALC. EGAVLPRSAK ELRCQCIKTY S.....KPFH
CON              A          A L   E    S S        CC  w SA   *k  P

           47                            72                            97
HHV-6 U83A  FRKLFDAIMI KKLKSCS.AA YPSDL.EQGS MC.DMADASP TSLELGLSKLDKES*
HHV-6 U83B  FRKPFVAIMI KKLKSCF.AA YLSDL.EQGS MC.DMANASP TSLELGLSKLDKES*
HHV-8 Mip1  QVLLSSW..Y PTSQLCSKPG VIFLTKRGRQ VCADKSKDWV KKLMQQLPVTAR*
RANTES      RAHIKEY..F YTSGKCSNPA VVFVTRKNRQ VCANPEKKWV REYINSL....EMS*
IL8         PKFIKELRVI ESGPHCANTE IIVKLSDGRE LCLDPKENWV QRVVEKFLKRAENS*
CON          k l d  mI       CS  A     L e    mC D  d          L K    S
```

FIG. 5. Analyses of strain variation and coding sequence in relation to the novel splicing. The splicing regulates chemokine expression via the introduction of a stop codon as the resulting encoded truncated peptide interrupts the chemokine consensus; this is shown in all strains analysed. Comparison of A and B variant strains with HHV-8 Mip1 homolog and prototype CC and CXC chemokines, Rantes and interleukin-8. CON indicates identical or similar (uppercase and lowercase) residues in U83 to chemokines. Conserved chemokine signature cysteines are underlined in the consensus. Splicing stop and termination codons are indicated by asterisks. U83 strain variations are underlined in U83B (Z29) compared with U83A (U1102). Strains analysed in Fig. 3 conform to A and B variants shown with minor additional variation as follows: HHV-6 B shown is for strains Z29, MBE, and HT; HHV-6B strains CV, BOU, Z3, Z12, Z13, Z14, Z25, and Z317 also have L27 to S; and strain CV also has S23 to F. HHV-6A is for strains U1102 and Z27. HHV-6A strains Z23 and Z59 also have P46 to S and M21 to I. HHV-6A Z5 also has A53 to E. HHV-6A strain Z23 also has I54 to V. HHV-6A strain Z59 also has M55 to L.

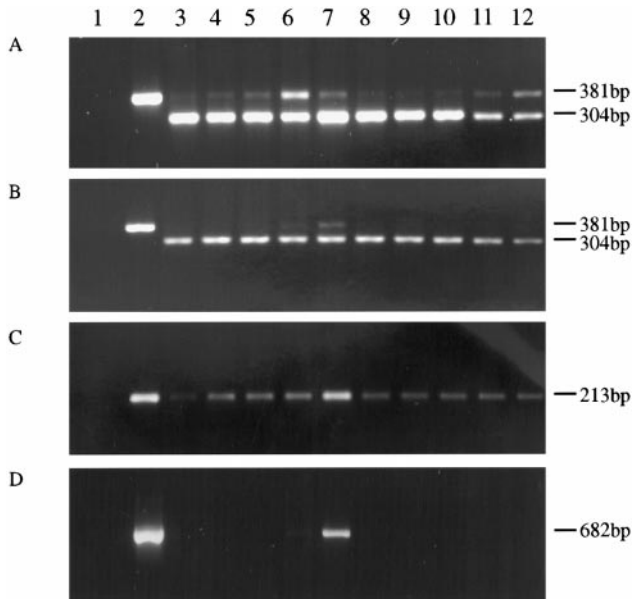

FIG. 6. Kinetics of expression for U83 RNA transcripts. (A) Strain U1102 RT-PCR products generated using U83 primers, U83–1 and U83–2, which amplify the entire U83 gene region. (B) As in A but using strain CV-infected cells. (C) Strain U1102 RT-PCR products generated using primers, U83–1 and U83INB, which are specific for the full-length transcript using one intron-specific primer; identical results were obtained with strain CV. (D) RT-PCR products generated using the U22 primers. RNA was isolated in the absence of inhibitors (lanes 3–7), and in the presence of CEX (lanes 8–10) and PAA (lanes 11 and 12). Lane 1, H_2O negative control; lane 2, U1102-infected DNA; lanes 3–12, HHV-6-infected cDNA at various time points p.i.; lanes 3 and 8, 3 h; lanes 4 and 9, 6 h; lanes 5 and 10, 8 h; lanes 6 and 11, 16 h; and lanes 7 and 12, 40 h. Sizes of products are indicated in base pairs.

then analysed using RT-PCR of cDNA generated from strain U1102- (variant A) and CV- (variant B) infected JJhan cells. Conditions were those established to define IE and late genes of HHV-6 as described previously (Mirandola *et al.,* 1998).

Figure 6 shows that both U83 transcripts occur early in infection and that neither transcript is inhibited by CEX, supporting grouping in the IE kinetic class. The spliced transcript (304 bp) is unaffected by the inhibitors CEX and PAA, suggesting regulation as an IE or E gene. Surprisingly, the full-length U83 transcript accumulated to higher levels at 16–40 h p.i. (Fig. 6A). This can be seen with greater clarity when the transcript is amplified using the same 5′ primer together with a 3′ intron-specific primer U83INB, which detects only the full-length transcript (Fig. 6C). The increase was reduced by blocking DNA replication with the inhibitor PAA. Comparisons were made with the other HHV-6-specific gene, U22. This had the kinetics of a true late gene in HHV-6, that is, a gene that is expressed at late times p.i. and is dependent on virus DNA replication for expression. U22 could be amplified at 40 h p.i. only, and its transcript was inhibited by PAA (Fig. 6D). No evidence for splicing of U22 was found by RACE and cDNA sequence analyses. Furthermore, in the absence of RT, no PCR product was obtained, indicating that the kinetics was due to RNA transcripts and not to DNA contamination, which may be introduced after the increased virus DNA replication at late times p.i. Thus the overall RT-PCR analysis demonstrated that the U83 spliced transcript displays some characteristics of IE kinetics, whereas the full-length transcript displays dual kinetics involving both early and late phases of infection. Similar results were obtained using the variant B strain CV, although the full length accumulated at later times (40 h), which was reduced by PAA (Fig. 6B). This may reflect a delayed replicative cycle or differing sensitivities to PAA. For all primer sets, no false-positive amplification was detected in uninfected cells or in the cell-free virus innoculum. Spliced products of U1102 and CV strains were confirmed by sequence analyses. These results indicated that the novel splice can be performed in the absence of virus protein production (CEX case), whereas the full-length product requires virus replication for maximal expression.

U83 novel splicing in a stably expressing cell line

Because the spliced product was expressed as an IE gene in the absence of virus protein expression, this result indicated that the novel splice can be recognised by cellular mechanisms. To further investigate this, a stable U83-expressing cell line was produced. The full-length U1102 U83 gene was amplified by PCR using primers U83P1 and U83P2, purified, and cloned into the eukaryotic expression vector pCDNA3, forming the construct pCDNA3-U83. This construct was transfected into the parental cell line and selected using G418. The external gene-specific primers used earlier were outside of the coding sequence amplified here, so internal U83 primers U83F2 and U83B2 were designed for RT-PCR amplification in the U83 cell line. Amplification of the cell line DNA resulted in a 233-bp product, whereas amplification of the cell line cDNA produced a band with re-

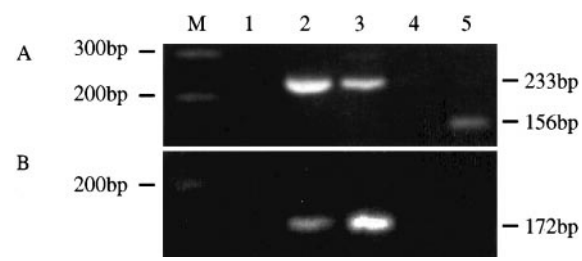

FIG. 7. U83 splicing in stable cell lines. (A) RT-PCR products generated using primers, U83F2 and U83B2, which amplify an internal portion of U83 and are designed to detect both spliced and unspliced products. (B) RT-PCR products using primers U83F2 and U83INB, designed to detect only the full-length transcript. Lane 1, H_2O negative control; lane 2, U1102-infected DNA; lane 3, HaCaT U83 DNA; lane 4, HaCaT U83 cDNA prepared without reverse transcriptase; and lane 5, HaCaT U83 cDNA prepared with reverse transcriptase. Sizes of products are in base pairs.

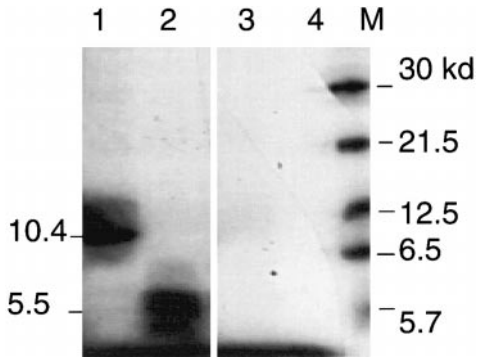

FIG. 8. *In vitro* transcription translation of U83 full-length and spliced genes. Lane 1 shows translated full-length U83; lane 2, spliced U83; lane 3, reverse orientation gene negative control; and lane 4, plasmid vector pCDNA3. Low-molecular-weight markers are indicated with sizes in kDa in lane M. The apparent molecular masses of translated full-length and spliced U83 are indicated as 10.4 and 5.5 kDa, respectively.

duced size, 156 bp, that corresponded to the spliced product in infected cells (Fig. 7A). However, under the conditions used here, the unspliced product was not detectable even using the intron primer (Fig. 7B) or two intron-specific primers (not shown). The RT-PCR product was gel-purified, and sequence analyses showed a spliced transcript of U83 identical to the spliced transcript in the infected JJhan cells and PBMCs (Fig. 2). This result demonstrates that there is recognition of the novel U83 splice donor and acceptor sites by cellular mechanisms in the absence of the virus, thus confirming the IE kinetic data that showed splicing in the absence of virus protein synthesis. It also shows that the splicing of U83 is not dependent on other viral factors or additional viral DNA components that are not included in the pCDNA3-U83 construct. Thus the novel cellular splicing stops expression of the encoded chemokine-like protein and virus regulation of this splicing allows the expression.

In vitro transcription translation analyses and immunofluorescence detection in infected cells

The full-length gene and spliced U83 cDNA were cloned in an expression vector and examined for protein expression. *In vitro* transcription translation showed that the full-length and spliced transcripts translated into 10.4- and 5.5-kDa proteins similar to the predicted molecular masses (Fig. 8). There was no evidence for RNA self-splicing in this system because the full-length transcribed product translated only into the predicted 10.4-kDa protein species. In studies to be described elsewhere, a GST U83 fusion protein was used to immunise mice, and a monoclonal antibody (MAb), FC2, was isolated from hybridoma cells using methods previously described (Liu *et al.,* 1993b). This antibody showed no reactivity to GST by immunoblotting and was specific for the fusion protein. In infected cells, the MAb was effective in immunofluorescence assays. Figure 9 shows specific reactivity to strain U1102-infected JJhan cells. In comparison with staining with HHV-6 gH MAb, the U83 MAb showed more diffuse cytoplasmic distribution with some evidence for surface capping as observed for gH. Positive immunofluorescence was observed 3–5 days p.i. with no reactivity to uninfected cells and no specific reactivity to the cell lines where only the spliced gene was detected. This supports the transcriptional analyses that show the gene is expressed during infection in tissue culture and indicates that protein levels are detectable at late times p.i., which would be consistent with production of the full-length transcript.

DISCUSSION

Novel splicing is demonstrated in the HHV-6-specific gene U83. Both in-frame full-length and spliced products are produced. The spliced product encodes a truncation of the full-length product. The splice is reproduced in a U83-expressing cell line, showing that this splicing can

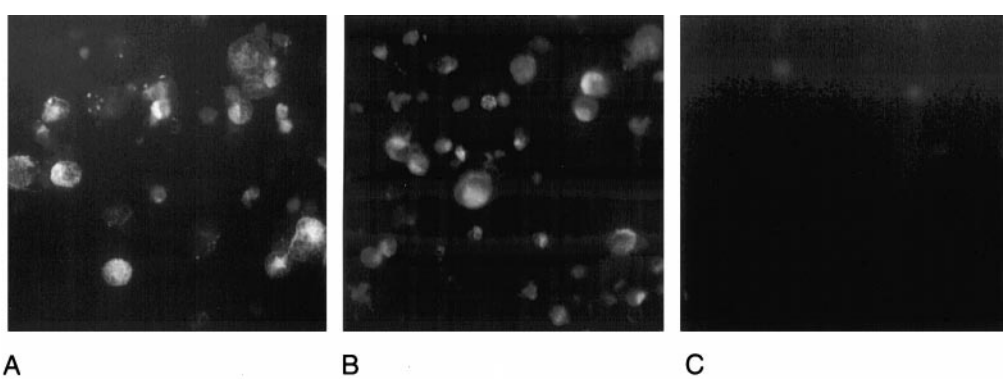

FIG. 9. Immunofluorescence staining of U83 MAb on HHV-6 strain U1102-infected JJhan cells. (A) Infected cells at day 4 p.i. stained with positive control MAb 2E4 specific for HHV-6 gH on acetone-fixed infected cells as described previously (Liu *et al.,* 1993b). (B) Staining with MAb FC2 specific for HHV-6 U83. (C) Negative control staining with MAb 52s specific for HSV gH (ATCC) (Gompels *et al.,* 1991). Using identical conditions, no reactions were observed against uninfected cells with these MAbs.

be cell mediated in the absence of other viral products. It is possible that this is also a new form of self-splicing, but the intron does not superficially resemble group I or II introns. Furthermore, T7 RNA polymerase-based *in vitro* transcription-coupled translation of the full-length gene translates only the complete full-length protein. The smaller product of the spliced gene can be detected only from *in vitro* transcription translation of cloned cDNA of the spliced transcript. The sites mediating the intron splicing appear to be novel and do not conform to major or minor class consensus sites for spliceosomal mediated mammalian mRNA splicing (Kramer, 1996). Moreover, the intron does not resemble the HAC1/ERN4 mRNA required for unconventional splicing involving tRNA ligase and Ire1p/Ern1p endonuclease in the unfolded protein response (Sidrauski and Walter, 1997; Kawahara *et al.,* 1998). In addition, given the conservation of sequences at the 5′ and 3′ splice sites and within strains, this suggests a different mode from tRNA splicing where sites are not conserved and tRNA splicing endonucleases measure from positions within the body of the mature RNA (Trotta *et al.,* 1997).

The U83 intron is bounded by a novel duplication TACC, which is extended in strain B variants to TACCTG. The existence of a duplication at these sites bears some resemblance to the AGG motif often duplicated at major splice junctions. If cleavage occurs in the same positions of the ACC motif as in the AGG motif (Fig. 3), then the U83 intron may represent a new minor class, CT-AC, in contrast to the major, GT-AG, and minor, AT-AC, spliceosomal classes. Alternatively, the duplication may suggest the use of a specific endonuclease. Recent results show the minor spliceosomal class may also use GT donors (Dietrich *et al.,* 1997), but the U83 splice sites still do not conform easily to this grouping. The major and minor classes use overlapping and distinct sets of snRNA molecules to mediate splicing to form similar spliceosomal machinery with differing specificity (Nilsen, 1996; Tarn and Steitz, 1996). Differences in base pairing with U11 and U12 snRNA, in particular, differentiate the minor class from U1- and U2-associated major class, and predictions were made on the basis of sequence comparisons (Hall and Padgett, 1994). In U83, these comparisons show that this intron does not fall easily into either group, possibly weak interactions are mediated by splicing enhancers and protein interactions, or this is a new class of splicing.

Studies presented here of the kinetics of expression of U83 suggest virus infection and replication affects this novel splicing in HHV-6 U83. In cells, only the spliced form could be detected. In virus-infected cells, both the full-length and spliced products are made with IE kinetics in the presence of translation inhibitor (CEX). The full-length product also further accumulated at late times p.i., and this accumulation was prevented using an inhibitor of virus DNA replication (PAA). In comparison, the HHV-6-specific gene U22 expressed only as a complete true-late gene, with expression dependent on virus DNA replication. Lack of the U83 full-length product in the cell line could also be due to differences in turnover or in the 5′ and 3′ untranslated regions in the cell-expressing construct, which lack, in particular, the 3′ AT-rich motifs found here in U83 and shown in other chemokine genes to regulate mRNA turnover (Lagnado *et al.,* 1994). Evidence for dual kinetics for different spliced transcripts from the same gene has also been shown for another HHV-6 IE gene, U91 (Mirandola *et al.,* 1998).

Viruses have been used as tools to investigate spliceosomal machinery and have shown roles for interactions between major and minor class introns in modulation of splicing efficiency. This is a cooperative mechanism involving many different components, including splice sites, intronic and exonic RNA sequences, snRNAs, snRNPs, and SR proteins, as reviewed by Kramer (1996). Studies have shown that SR proteins and enhancer elements, often purine-rich elements (PREs), participate in splicing regulation (Manley and Tacke, 1996). HSV has been shown to alter the activity or distribution of SR proteins (Martin *et al.,* 1984), and nonconsensus splice donors (GC) have been identified in latency transcripts that can be involved in alternative spliced transcripts (Spivack *et al.,* 1991; Sandri-Goldin *et al.,* 1995). There are numerous examples of sequences in both introns and exons that act to inhibit or promote splicing (Cote *et al.,* 1992; McNally and Beemon, 1992; Amendt *et al.,* 1994, 1995; Tanaka *et al.,* 1994; Zhao *et al.,* 1994; Staffa and Cochrane, 1995). Regulation sequences, such as PREs, are frequently associated with suboptimal 3′ splice sites, such as those seen in U83. Moreover, the exon-splicing silencer element of the HIV-1 tat-rev exon was able to efficiently inhibit splicing in conjunction with suboptimal 3′ splice sites, such as itself and the Rous sarcoma virus src, but not with consensus splice sites that occur in the β-globin intron and the fibronectin gene (Amendt *et al.,* 1994; Staffa and Cochrane, 1995). Splicing enhancer elements and SR proteins of the adenovirus L1 unit have been shown to act as negative regulators of the alternative 3′ IIIa splice site by inhibiting the recruitment of the U2 small nuclear ribonucleoprotein particle where the position of the PRE in relation to the 3′ splice site determines whether the enhancer acts positively or negatively (Kanopka *et al.,* 1996). It has recently been demonstrated that the E4-ORF4 adenovirus protein is responsible for regulating the shift in L1 alternative splicing at the late phase of virus infection (Kanopka *et al.,* 1998). This involved an E4-ORF4-kinase complex in the dephosphorylation of SR proteins, which prevented the inhibitory effect and allowed splicing at the distal IIIa splice site. *In vitro* studies have also shown that protein kinases are capable of directly inhibiting splicing (Gul *et al.,* 1994). Recent data show that in a retrovirus, an enhancer-like PRE sequence is a component of a splicing in-

hibitor element (McNally and McNally, 1998). It is postulated that this control involves interaction between major and minor splice sites. It is possible that such a scenario may apply to HHV-6 U83 in that there are predicted 5′ major donor sites that are not utilised and the entire intron is purine rich, which may resemble a PRE. Analyses of the HHV-6 genome sequence identified three potential SR proteins, U86, DR8, and DR1 (Gompels *et al.,* 1995), and they may participate in such control of RNA processing efficiency.

The splicing in U83 regulates expression of the chemokine-like protein by introducing a termination codon that stops expression of the protein encoded by the full-length transcript. The splicing seems to be efficient and functioning before the expression of any virus proteins (CEX treatment). Although U83 is relatively variable (13%) between HHV-6 strains, the novel splice site is conserved. The high variation in U83 is interesting in that the adjacent genes, such as glycoprotein gL, are conserved and that there is a high proportion of coding changes in U83 indicative of some selection, possibly immune driven or via different ligand–receptor interactions. However, splicing efficiency and the structures of both the full-length and truncated U83 protein, produced by a spliced stop codon, are all conserved between strains. This seems to be a general mechanism for the control of this HHV-6-specific gene product using a cellular splicing pathway. HHV-6 infects cells of hemapoietic origin; thus the chemokine-like U83 protein may affect virus spread in mediating an inflammatory response that could be critical to lytic or latent infections by this virus. In another herpesvirus, murine cytomegalovirus, which also encodes a protein with chemokine sequence similarity, the transcript is regulated as a late gene (MacDonald *et al*., 1997). In HHV-6, the full-length U83 is also regulated as a late gene, whereas the spliced and interrupted transcript has characteristics of an IE gene. The truncated peptide encoded in the spliced message interrupts the similarity with the chemokine family.

In summary, U83 shows novel splicing that can be accomplished in cells but can be affected by virus infection. The splicing regulates expression of the encoded chemokine-like protein by introducing a spliced stop codon interrupting the gene, whereas the full-length product is observed only after virus infection and replication. This novel regulation of HHV-6 U83 may also indicate a distinct pathway for cellular control of gene expression.

MATERIALS AND METHODS

Cells and virus

The $CD4^+$ T lymphocyte cell line JJhan (Jurkat clone, T-leukemic) and PBMCs were grown in suspension culture at 37°C in RPMI containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) FCS. Viral cultures were prepared through infection of JJhan cells or PBMCs with HHV-6 strain U1102, variant A (Gompels *et al.,* 1995), or CV, variant B, as described previously (Mirandola *et al.,* 1998). HaCaT human epithelial cell lines were grown in monolayers, in identical media, and derived transfectants were cultured with the addition of 500 μg/ml G418. Cellular and viral DNA were extracted from 5-day-old cultures using a proteinase K–SDS, phenol–chloroform extraction procedure.

PCR analysis of DNA

Isolation of DNA from HHV-6-positive Zambian patient samples has been described by one of our groups previously (Kasolo *et al.,* 1997); the DNA was kindly donated by F. C. Kasolo (University Teaching Hospital, Zambia; London School of Hygiene and Tropical Medicine, UK). Z29 DNA was kindly provided by Prof. G. Campadelli-Fiume (University of Bologna, Bologna, Italy). Sample DNA was amplified for the U83 gene using primers U83P6 (5′-ACAAATAGTAAAGGATCCATGGTT-3′) and U83B (5′-CGACAATCCTAATTCAAGAC-3′). PCR was carried out under standard conditions with 1.5 U of *Taq* polymerase and buffer (Promega, Madison, WI), 1 μM concentration of the primers, 200 μM concentration of the dNTPs, and 1.5 mM $MgCl_2$ in a total volume of 29 μl with 1 μl of template or control DNA. The thermocycling procedure was 30 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. Each run contained a reagent (water), negative control (uninfected JJhan cellular DNA), and positive control (U1102-infected JJhan cellular DNA or plasmid pCDNA3-U83 DNA). The PCR products were resolved and detected visually by 2.0% agarose gel electrophoresis with ethidium bromide staining.

RT-PCR analysis of RNA

Cells from confluent cultures were washed twice in sterile PBS. Total cellular RNA was extracted using RNA Isolator (Genosys, Cambridge, UK), where the RNA pellet from 10 ml of starting culture was resuspended in a total volume of 50 μl. For each sample, a 25-μl aliquot was stored at −80°C, and the remainder was treated with 2.5 U of DNase RQ1 (Promega) to remove residual DNA before first-strand cDNA synthesis. First-strand cDNA synthesis was performed as follows: 2 μg of total RNA and 1 μM poly(dT) adapter primer (GIBCO BRL, Gaithersburg, MD) in a final volume of 11 μl was incubated at 65°C for 10 min, followed by 10 min on ice. This RNA was reverse transcribed in a final volume of 50 μl containing 40 U of Moloney murine leukaemia virus RT (Promega), 10 mM dithiothreitol, 500 μM dNTP, and 40 U of RNasin inhibitor (Promega) for 1 h at 37°C, followed by 10 min on ice. The cDNA was amplified by PCR under standard

conditions (see PCR analysis of DNA), using 5 μl cDNA template and gene-specific primers for U83: U83P7 (5′-ACGGGATCCACATCTTATATATAG-3′) with U83C (5′-TAATGTCTTCTAGATACTTTC-3′) or U83F2 (5′-CCCCGATGCGGAGCTGTTTTCC-3′) with U83B2 (5′-CAAGACTTGTCGGCGATGCATC-3′).

In addition, F2 was used with an intron-specific 3′ primer, U83INB (5′-GGTAAGCAGCAGAACAAC-3′).

Actin primers were used as internal controls: actin-F (5′-GATGGAGTTGAAGGTAGTTT-3′) and actin-B (5′-TGCTATCCAGGCTGTGCTAT-3′).

All samples were checked for DNA contamination and were PCR negative in the absence of RT. RACE was performed under standard conditions for 40 cycles, using 5 μl sscDNA template and the gene-specific primer (U83F2) with the poly(dT) adapter primer (GIBCO BRL). A positive control for the 3′ RACE reaction was performed using control RNA obtained from GIBCO BRL. PCR products were separated by 2% agarose electrophoresis and visualised by ethidium bromide staining. cDNA was gel purified using GeneClean (Anachem) and either sequenced directly or cloned into the TA cloning vector pGEM^{R}-T (Promega) before sequencing.

DNA sequencing

HHV-6 DNA PCR products were gel purified using GeneClean. Plasmid DNA of clones were prepared using SV Wizard preps (Promega). Sequencing reactions were carried out using the ABI Prism Dye terminator cycle sequencing ready reaction kit (Perkin–Elmer Cetus, Norwalk, CT). DNA was sequenced on the ABI Prism 377 sequencer (Perkin–Elmer).

Kinetics of expression

Time course infection and metabolic inhibition studies with CEX, to inhibit protein synthesis, and PAA, to inhibit viral DNA replication, were done essentially as described before using both strains U1102 (HHV-6 variant A) and CV (HHV-6 variant B) (Mirandola *et al.,* 1998). Cell-free virus was used to inoculate cell cultures, and RNA was harvested at intervals p.i. in the presence or absence of CEX or PAA acid treatment as described previously (Mirandola *et al.,* 1998). RT-PCR analyses were performed using reverse-transcribed poly(A)$^{+}$-selected mRNA with random hexamer primers to generate cDNA followed by PCR with the following primers for U83, detecting full-length and spliced products 83–1 (5′-CGGCATATATTGGTATGGCT-3′) and 83–2 (5′-TTGGTCTCTTTGCGTCTTCT-3′); for the full-length product primer 83–1 together with the intron-specific primer U83INB (as above); and for gene U22, 22–1 (5′-GTGATTATGGTGCCTCAGGG-3′) and 22–2 (5′-GATCGCCGTCATACCACAAC-3′).

Construction of U83 expressing cell line

The U83 gene was amplified using PCR with standard thermocycling procedures and *Taq* polymerase (Promega). The following primer set was used to amplify the coding region and to introduce *Bam*HI restriction enzyme recognition sequences: U83P1 (5′-ACGGGATCCATTGGTATGGCT-3′) and U83P2 (5′-TTCGGATCCTTTCATGATTCT-3′). The product amplified was then digested with *Bam*HI restriction enzyme, separated by agarose gel electrophoresis, and purified by phenol extraction followed by ethanol precipitation. The purified DNA was ligated with *Bam*HI-digested, alkaline phosphatase-treated (Boehringer-Mannheim Biochemica, Mannheim, Germany) plasmid pCDNA3 (InVitrogen, San Diego, CA) and transformed into *Escherichia coli* strain JM109. The selected clone was checked by sequence analyses in comparison with published sequence and separately amplified products from infected cell DNA. The plasmid with the gene oriented for expression from the CMV promoter was designated pCDNA3-U83. Both vector-only, pCDNA3, or U83 cloned DNA, pCDNA3-U83, were used to transfect human epithelial cells (HaCaT) using Lipofectin (Life Technologies, Eggenstein, Germany) according to the manufacturer's instructions. Expressing colonies were selected and grown using culture media (RPMI, 10% FCS with supplements as above) with 500 μg/ml G418 (GIBCO BRL). Colonies were cloned using perspex rings and analysed using the SP6 and T7 primers to amplify across the multiple cloning site in DNA PCR; expression was confirmed using RT-PCR and specific primers. Sequence analyses showed conservation of the U83 sequence.

In vitro transcription translation analyses

The cDNA from the spliced U83 was similarly cloned as described above by ligation with the pCDNA3 plasmid vector. *E. coli* JM109 was transformed with either the full-length and spliced U83 plasmids and DNA prepared using Quiagen minipreps. *In vitro* transcription and translation was performed using the TNT Coupled Rabbit Reticulocyte Lysate system (Promega) according to the manufacturer's instructions. Briefly, 0.5 μg of plasmid DNA was used in an *in vitro* transcription and translation mix containing 12.5 μl of rabbit reticulocyte lysate, 20 U of RNasin, 15 μCi of ^{35}S-methionine (SJ1515; Amersham Corp., Paisley, UK), and 7.5 U of T7 RNA polymerase. Reactions conditions used were 30°C for 90 min, followed by separation of translated products on SDS–PAGE with 18% acrylamide gels (Novex, San Diego, CA).

Immunofluorescence

As described previously (Liu *et al.,* 1993a), U1102-infected JJhan cells were fixed with acetone, washed in PBS, blocked with neat FCS, reacted with monoclonal

antibodies for 30 min at 37°C, further washed in PBS, and then reacted with fluorescein isothiocyanate conjugated to rabbit anti-mouse IgG (1:200; Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. After PBS washes, the cells were examined by fluorescence microscopy with 400× magnification and 450/410-nm wavelength.

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